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Identification of Tocopherols, Tocotrienols, and Their Fatty Acid Esters in Residues and Distillates of Structured Lipids Purified by Short-Path Distillation

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ABSTRACT: The fate of endogenous vitamin E isomers during production and purification of structured lipids (SLs) was investigated. Two SLs involving tripalmitin, stearidonic acid soybean oil, and docosahexaenoic acid were synthesized by transesterification catalyzed by Novozym 435 (NSL) and acidolysis by Lipozyme TL IM (LDHA) and purified by short-path distillation (SPD). The electron impact and chemical ionization mass spectra of tocopheryl and tocotrienyl fatty acid esters in the distillates measured by GC-MS in synchronous scan/SIM mode demonstrated that these esters were formed during acidolysis as well as transesterification. The predominant esters were tocopheryl palmitate, tocopheryl oleate, and tocopheryl linoleate homologues, and no tocopheryl or tocotrienyl linolenate, stearidonate, or docosahexaenoate was found. Meanwhile, none of these esters were detected in the residues for either NSL or LDHA. Less than 50% of vitamin E isomers were present in residues after SPD. This loss played a major role in the rapid oxidative deterioration of SLs from previous studies with less contribution from the formation of tocopheryl and tocotrienyl esters. The lost tocopherols and tocotrienols present at high concentration in the distillates may be recovered and used to improve the oxidative stability of SLs.

KEYWORDS: *tocopherol, tocotrienol, tocopheryl/tocotrienyl fatty acid ester, short-path distillation, oxidative stability, structured lipid, stearidonic acid soybean oil*

INTRODUCTION

Structured lipids (SLs) are generally defined as triacylglycerols (TAGs) that have been chemically or enzymatically modified from their natural biosynthetic state by changing the fatty acid (FA) composition and/or the positions of FAs in the glycerol backbone to yield novel lipids. Lipase-catalyzed synthesis of SLs, commonly transesterification and acidolysis, is preferred over chemical synthesis because of specificity and better control over the final products.[1] The current popularity of SLs is well-known because of their desired physical characteristics, chemical properties, and/or health benefits, for food or nutritional applications,[2] such as human milk fat analogues, reduced-calorie fats, enteral and parenteral nutrition, *trans*-free margarines, and cocoa butter substitutes. Furthermore, it is important to consider whether such tailor-made lipids are oxidatively stable to allow their use as ingredients. The stability of fats and oils depends on a number of intrinsic and extrinsic factors, mainly the unsaturation degree of FAs, minor components, environmental conditions (e.g., oxygen, light, and temperature), and use of antioxidants.[3]

Tocopherols and tocotrienols, naturally occurring minor components present in vegetable oil, are known as important endogenous antioxidants that protect oil against oxidation. It is widely accepted that the antioxidant activities of the tocopherols and tocotrienols are mainly due to their abilities to donate their phenolic hydrogens to lipid free radicals and retard lipid peroxidation process. The relative antioxidant activities of the tocopherols in vivo are α- > β- > γ- > δ-tocopherol.[4] However, relative antioxidant activities in model and food systems are variable. α-Tocopherol generally was a more effective antioxidant at low concentration but a less effective antioxidant at high levels than γ-tocopherol in fats and oils.[5] γ-Tocotrienol was found to have higher antioxidant activity than α-tocotrienol, and tocotrienols may be better antioxidants than their corresponding tocopherols in certain fat and oil systems.[5]

Modified TAGs, free fatty acids (FFAs), or fatty acid esters are the main components of products after lipase-catalyzed reactions. FFAs and fatty acid esters should be removed from the products before they are used for edible purposes. Modified TAGs, especially those containing polyunsaturated fatty acids, are heat sensitive and have high boiling points. Therefore, thermal decomposition and oxidation easily take place during purification at high temperatures under atmospheric pressure. Furthermore, severe acyl migration may occur under harsh purifying conditions due to the presence of diacylglycerols in the reaction mixture, which act as intermediates for the lipase-catalyzed reactions.[6] These limitations can be minimized by use of short-path distillation (SPD). SPD, also called molecular distillation, is a thermal separation technique based on an apparatus with a gap between the evaporator and condenser of equal or less dimension than the mean free path of the molecules to be evaporated.[7] It is characterized by the combination of very short residence time in the evaporator (1−10 s), high vacuum level (0.1−100 Pa), short distance between the evaporator and condenser (10−50 mm), and approximately collision-free mass transfer of molecules in the distillation space.[8,9] Samples are fractionated into a heavy fraction (residue) and a light fraction (distillate or waste) on

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Table 1. Total Fatty Acid Profiles (Mole Percent) and Molecular Weight of Substrates and Scaled-up SLs at Optimal Conditions[a]

fatty acid	SDASO (866.74)[b]	DHASCO-FFAs (287.46)	NSL (827.76)	LDHA (835.83)
C8:0	ND	0.46 ± 0.00	ND	ND
C10:0	ND	1.74 ± 0.02a	ND	0.38 ± 0.01b
C12:0	ND	5.48 ± 0.05a	ND	1.31 ± 0.01b
C14:0	0.10 ± 0.00d	10.94 ± 0.10a	1.42 ± 0.00c	3.60 ± 0.01b
C14:1	ND	0.25 ± 0.00a	ND	0.06 ± 0.00b
C16:0	13.93 ± 0.03c	7.45 ± 0.08d	67.49 ± 0.12a	55.94 ± 0.45b
C16:1n-7	0.11 ± 0.00c	3.21 ± 0.01a	0.05 ± 0.01d	0.78 ± 0.01b
C17:0	0.08 ± 0.07a	ND	0.07 ± 0.01b	0.07 ± 0.00b
C18:0	3.87 ± 0.04a	0.72 ± 0.02d	2.54 ± 0.01b	2.14 ± 0.00c
C18:1n-9	13.21 ± 0.15b	23.07 ± 0.12a	5.04 ± 0.01d	9.40 ± 0.16c
C18:2n-6	24.22 ± 0.07a	1.17 ± 0.00d	8.51 ± 0.02b	7.26 ± 0.09c
C20:0	0.21 ± 0.00a	ND	0.08 ± 0.00b	0.07 ± 0.00c
C18:3n-6	7.22 ± 0.03a	ND	2.44 ± 0.01b	2.29 ± 0.03c
C20:n-91	0.17 ± 0.00a	ND	0.07 ± 0.00b	0.07 ± 0.00b
C18:3n-3	11.01 ± 0.07a	ND	3.71 ± 0.02b	3.06 ± 0.04c
C18:4n-3	25.74 ± 0.14a	ND	8.39 ± 0.03b	7.97 ± 0.07c
C22:0	0.11 ± 0.00a	ND	0.04 ± 0.00b	0.05 ± 0.01b
C24:0	0.03 ± 0.00a	ND	0.01 ± 0.00b	0.01 ± 0.00b
C22:6n-3	ND	45.51 ± 0.38a	ND	5.43 ± 0.06b

[a]Mean ± SD, $n = 3$. Values with different letters in the same row are significantly different by Duncan's multiple-range test ($P < 0.05$). Abbreviations: SDASO, stearidonic acid soybean oil; DHA, docosahexaenoic acid; DHASCO-FFAs, free fatty acids hydrolyzed from DHA single-cell oil; NSL, structured lipid of SDASO/tripalmitin (1:2, mol/mol) at 65 °C for 18 h catalyzed by 10% Novozym 435; LDHA, structured lipid of DHA/NSL (1:1, mol/mol) at 65 °C for 24 h catalyzed by 10% Lipozyme TL IM; ND, not detected. [b]Average molecular weight (in parentheses), $n = 3$.

the basis of volatility. SPD lowers the boiling temperature and is an excellent method for the separation, purification, and concentration of thermolabile substances with low vapor pressure. There are many applications of SPD in lipids, such as purification of SLs,[10] fractionation of fatty acid ethyl esters,[11] and concentration of squalene from shark liver oil.[12]

Several authors[13−16] have reported that SLs produced by both lipase-catalyzed transesterification and acidolysis are characterized by a lower oxidative stability compared to the initial fat/oil substrates. This decrease may be due to exposure to light, oxygen, and high temperature during reaction and purification steps (e.g., distillation, washing, or filtration). The most serious concern is the loss of endogenous antioxidants during production and purification, especially tocopherols and tocotrienols, when vegetable oils are used as substrates for the production of SLs. Hamam and Shahidi[17] reported that tocopherols in the oils were esterified with free fatty acids present at high level in the reaction mixture during production of SLs by enzymatic acidolysis, thus leading to the formation of tocopheryl esters that do not provide any stability to the resultant modified oils as they lack any free hydroxyl groups on the phenolic ring of the molecule. These tocopheryl esters may be removed from the reaction product during SPD. However, the mechanism of tocopherol loss associated with transesterification, another important reaction for the production of SLs with much lower content of FFAs, is unknown. For the purification step, many studies[10,13,14,18] reported that substantial amounts of tocopherols and tocotrienols were lost during SPD of SLs while decreasing the FFA content. However, few of them indicated where these tocopherols and tocotrienols were after SPD. These vitamin E isomers may be recovered and added back to SLs to compensate for their oxidative stability. Therefore, the first objective of this study was to investigate the removal or loss of endogenous antioxidants during lipase-catalyzed transesterification by identifying tocopheryl and/or tocotrienyl fatty acid esters present in residues and distillates of SLs purified by SPD. Lipase-catalyzed acidolysis was also examined to verify previous findings.[17] Our second objective was to quantitatively determine tocopherols and tocotrienols present in all of the fractions after SPD and to find a possible source of reusable tocopherols and tocotrienols.

MATERIALS AND METHODS

Materials. Stearidonic acid soybean oil (SDASO) was kindly donated by Monsanto Co. (St. Louis, MO, USA). Docosahexaenoic acid single-cell oil (DHASCO) containing 40% DHA was purchased from Martek Bioscience Corp., now DSM Nutritional Products Ltd. (Columbia, MD, USA). *sn*-1,3 specific lipase Lipozyme TL IM and nonspecific lipase Novozym 435 were obtained from Novozymes A/S (Bagsvaerd, Denmark). Supelco 37 component FAME mix, 2,5-dihydroxybenzoic acid, and *α*-, *β*-, *γ*-, and *δ*-tocopherols were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Methyl nonadecanoate was obtained from Nu-Chek Prep, Inc. (Elysian, MN, USA), and stearidonic acid methyl ester was purchased from Cayman Chemical (Ann Arbor, MI, USA). Tripalmitin and nonadecanoic acid were purchased from TCI America (Portland, OR, USA). Vitamin E linoleate mixture, consisting of 50−65% tocopheryl linoleate, 25−40% tocopheryl oleate, and no more than 1% *α*-tocopherol, was generously provided by TRI-K Industries, Inc. (Denville, NJ, USA). All solvents were of analytical grade and purchased from J. T. Baker Chemical Co. (Phillipsburg, NJ, USA) or Fisher Scientific (Norcross, GA, USA).

Scaled-up Synthesis of SLs. Two SLs were respectively scaled up in a 1 L stir-batch reactor with constant stirring at 200 rpm using optimal conditions previously reported.[16,18] Briefly, structured lipid of SDASO and triplamitin catalyzed by 10% Novozym 435 (NSL) was produced through transesterification at 65 °C for 18 h with a substrate mole ratio of 1:2. Structured lipid of NSL and DHASCO-FFAs catalyzed by 10% Lipozyme TL IM (LDHA) was prepared through acidolysis at 65 °C for 24 h with a substrate mole ratio of 1:1. DHASCO-FFAs were prepared from DHASCO by saponification as described by Teichert and Akoh.[18] The reactor was sealed and wrapped with foil to reduce exposure to light and oxygen. After

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reaction, the resulting products were vacuum filtered through a Whatman no. 1 paper filter containing anhydrous sodium sulfate to dry and separate the SLs from the biocatalyst. If the products needed to be stored before SPD, they were flushed with nitrogen and kept in an airtight amber container at −80 °C.

Short-Path Distillation. SPD was used to remove FFAs from the reaction mixture. SPD was performed using a KDL-4 (UIC Inc., Joliet, IL, USA) system under the following conditions: feed holding temperature of 65 °C for NSL and LDHA, feeding rate of approximately 100 mL/h, evaporator heating temperature of 185 °C, condenser cooling temperature of 20−25 °C, roller speed of 200 rpm, and vacuum of <13.33 Pa. SLs were passed through SPD once (NSL) or twice (LDHA) to obtain FFA concentrations of <1%. FFA content expressed as oleic acid percentage was determined according to AOCS Official Method Ca 5a-40.[19] The average and standard deviation of triplicate analyses were reported (Table 2).

Fatty Acid Composition. Lipid samples were converted to FAMEs following AOAC Official Method 996.01[20] with minor modifications. Briefly, 100 mg of sample was weighed into a Teflon-lined test tube, and 100 μL of 20 mg/mL C19:0 in hexane was added as an internal standard and dried with nitrogen to remove the solvent. Two milliliters of 0.5 mol/L NaOH in methanol was added, followed by vortex and incubation at 100 °C for 5 min to saponify the lipid. After cooling under the tap water, 2 mL of 14% boron trifluoride in methanol was added. Then the sample was vortexed for 1 min, incubated at 100 °C for 5 min for methylation, and cooled under tap water. To stop the reaction and extract the FAMEs, 2 mL of hexane and 2 mL of saturated NaCl solution were added. The sample was vortexed for exactly 2 min and then centrifuged at 2000 rpm for 5 min to separate the organic and aqueous layers. The upper organic layer was filtered through an anhydrous sodium sulfate cartridge and recovered into a GC vial for analysis. FAME external standard mixture containing Supelco 37 component FAME mix, methyl nonadecanoate, and stearidonic acid methyl ester was run parallel with the sample.

FAME samples were analyzed with an Agilent 6890N GC (Agilent Technologies Inc., Santa Clara, CA, USA) equipped with an autosampler and a flame ionization detector. Separation was achieved on a 100 m × 0.25 mm i.d., 0.20 μm film thickness, Supelco SP-2560 fused silica capillary column (Sigma-Aldrich Chemical Co.). The temperature program was 140 °C initially for 5 min, ramped to 240 °C at 4 °C/min, and held for 20 min. Helium was the carrier gas at a constant flow rate of 1.1 mL/min. Split injection at a ratio of 5:1 was employed, and the injection volume was 1 μL. The injector and detector were maintained at 260 °C. Relative fatty acid content as mole percent was calculated on the basis of response factors and conversion factors,[20,21] using C19:0 as internal standard. The average and standard deviation of triplicate analyses were reported (Table 1).

Determination of Tocopherols and Tocotrienols by HPLC. Lipid samples (0.1 ± 0.001 g) were weighed into a test tube, and 5 mL of HPLC-grade hexane was added. The samples were then vortexed for 30 s and centrifuged at 1000 rpm for 10 min. A portion of the hexane layer was transferred into an HPLC vial for analysis. Tocopherol standards were prepared according to the procedure of previous studies.[16,22,23] Twenty microliters of the sample extract or tocopherol standard solution was injected into a HPLC system consisting of a Shimadzu LC-6A pump equipped with an RF-10A$_{XL}$ fluorescence detector (Shimadzu Corp., Columbia, MD, USA), a SpectraSeries AS 3000 autosampler (Thermo Separation Products Inc., San Jose, CA, USA), a 250 mm × 4 mm i.d., 5 μm particle size, normal phase LiChrosorb Si 60 Hibar RT column (Merck KGaA, Darmstadt, Germany), and an Agilent Chemstation software (Agilent Technologies Inc.). The isocratic mobile phase contained 0.85% (v/v) isopropanol in hexane, and the flow rate was 1.0 mL/min. The excitation and emission wavelengths of the detector were 290 and 330 nm, respectively. Quantitation of tocopherols was performed using an external standard calibration curve method. Palm olein was used to identify α-, γ-, and δ-tocotrienols.[19] The fluorescence intensity of tocotrienols is the same as the corresponding tocopherols, so the tocotrienol content may be calculated using the same calibration curve of the corresponding tocopherols according to AOCS Official Method Ce 8-89.[19] All samples were analyzed in triplicate, and average values and standard deviation were reported (Table 2).

Without considering the decomposition by light, oxygen, and heat, the loss percentage of tocopherol or tocotrienol after reaction was calculated on the basis of the law of conservation of mass, using the equation

$$\begin{aligned}&\text{loss (\%) after reaction}\\&= [MW_a \times mol_a \times C_a + MW_b \times mol_b \times C_b\\&\quad - (MW_a \times mol_a + MW_b \times mol_b) \times C_{SL}]/\\&\quad (MW_a \times mol_a \times C_a + MW_b \times mol_b \times C_b) \times 100 \qquad (1)\end{aligned}$$

where MW_a and MW_b are the molecular weights of substrates a and b, respectively; mol_a and mol_b are the mole units of substrates a and b, respectively; C_a, C_b, and C_{SL} are the tocopherol or tocotrienol contents (μg/g) of substrates a, b, and structured lipid after reaction, respectively.

Similarly, the loss percentage of tocopherol or tocotrienol after SPD was calculated as

$$\text{loss (\%) after SPD} = \frac{(C_{SL} - C_R) \times C_W}{(C_W - C_R) \times C_{SL}} \times 100 \qquad (2)$$

where C_W, C_R, and C_{SL} are the tocopherol or tocotrienol contents (μg/g) of distillate or waste after SPD, residue after SPD, and structured lipid after reaction, respectively.

Tocopheryl and Tocotrienyl Esters Analysis by MALDI-TOF-MS. Lipid samples were dissolved in isopropanol at 1 mg/mL. A 20 mg/mL mixture of 2,5-dihydroxybenzoic acid in 50% methanol was used as matrix. For MALDI analysis, 0.8 μL of sample and matrix, respectively, was mixed together in a ratio of 1:1 (v/v) on a stainless steel MALDI target and then air-dried at room temperature. Samples were analyzed on a TOF/TOF 5800 system (AB Sciex, Framingham, MA, USA) equipped with a nitrogen laser (λ = 337 nm) and a reflector detector. An acceleration voltage of 20 kV was used. Mass spectra within an m/z range of 300−900 were acquired in positive ion mode. A magnified image of the MALDI target was used to visually select regions of sample. Spectra were acquired with adjustment of the sample position to produce intense ions for tocopheryl and tocotrienyl esters.

Tocopheryl and Tocotrienyl Esters Analysis by GC-MS. Two ionization modes, EI and PCI, were employed to identify tocopheryl and tocotrienyl esters. GC-MS measurements operated in the EI mode was performed on an Agilent 7890 GC coupled to a 5975C MSD and an autoinjector (Agilent Technologies Inc.). A 30 m × 0.25 mm i.d., 0.25 μm film thickness, Alltech Econo-Cap EC-1 fused silica capillary column (Alltech Associates Inc., Deerfield, IL, USA) was used for separation. The column temperature was maintained at 80 °C for 2 min, then increased at 30 °C/min to 140 °C, and held for 2 min, finally programmed to 325 °C at 20 °C/min, and held isothermally at 325 °C for 70 min (total 85.25 min). Carrier gas was helium with purity above 99.999%, and the flow rate was set at 1 mL/min. The injector temperature was maintained at 290 °C. Splitless injection was employed, and the injection volume was 2 μL. MS detection was performed on a single-quadrupole mass spectrometer under synchronous scan/SIM mode, and conditions were as follows: ion source, 230 °C; electron energy, 70 eV; transfer line, 300 °C; quadrupole,150 °C; m/z range, 30−780. Characteristic ions in the SIM mode were m/z 430, 416, 416, and 402 for α-, β-, γ-, and δ-tocopheryl esters, respectively, and m/z 424, 410, 410, and 396 for α-, β-, γ-, and δ-tocotrienyl esters, respectively. Dwell time was 100 ms each. The PCI mass spectra in synchronous scan/SIM mode were acquired on a Shimadzu GCMS QP2010 Ultra system, using ammonia as the reagent gas. Other chromatographic conditions were identical to those described for the EI mode.

Statistical Analysis. One-way ANOVA was conducted using SAS software, version 9.2 (SAS Institute Inc., Cary, NC, USA). Duncan's multiple-range test was performed to determine significant differences of variables (α = 0.05).

Table 2. Tocopherol and Tocotrienol Concentration and Free Fatty Acid Percentage of Substrates and Scaled-up SLs[a]

	tocopherol and tocotrienol contents (μg/g)							FFA (%)
	α-T	α-T_3	β-T	γ-T	γ-T_3	δ-T	total	
NSL								
SDASO (Sr)	63.01 ± 1.82b	ND	10.82 ± 0.93b	565.83 ± 4.83b	ND	192.20 ± 5.52b	831.87 ± 13.09b	0.02 ± 0.00b
NSL before SPD	20.88 ± 1.35c (5%C)	ND	3.48 ± 0.08c (8%A)	195.11 ± 8.28c (1%D)	ND	62.57 ± 2.69c (7%B)	282.04 ± 12.41c (3%)	2.17 ± 0.03a
NSL after SPD	10.20 ± 0.28d (52%C)	ND	1.73 ± 0.18d (51%D)	87.14 ± 0.20d (57%B)	ND	20.73 ± 0.16d (68%A)	119.81 ± 0.82d (59%)	0.03 ± 0.01b
W_{NSL} after SPD	694.03 ± 26.35a	ND	155.94 ± 5.49a	4170.62 ± 66.92a	ND	1890.31 ± 12.70a	6910.9 ± 47.78a	NA
LDHA								
DHASCO-FFAs (Sr)	26.76 ± 0.28b	174.60 ± 2.22a	6.09 ± 0.17a	72.64 ± 1.73c	4.74 ± 0.12a	38.04 ± 3.89b	322.87 ± 8.42b	NA
NSL after SPD (Sr)	10.20 ± 0.28c	ND	1.73 ± 0.18d	87.14 ± 0.20b	ND	20.73 ± 0.16c	119.81 ± 0.82d	0.03 ± 0.01c
LDHA before SPD	10.57 ± 0.22c (27%B)	40.46 ± 0.48c (10%D)	2.28 ± 0.01c (20%C)	61.05 ± 0.53d (27%B)	0.63 ± 0.05b (48%A)	20.24 ± 0.16c (20%C)	135.23 ± 1.44c (21%)	26.95 ± 0.02a
LDHA after SPD	5.23 ± 0.09d (61%D)	28.93 ± 0.53d (39%E)	ND (100% A)	13.92 ± 0.20e (86%C)	ND (100% A)	3.75 ± 0.02d (88%B)	51.82 ± 0.84e (73%)	0.35 ± 0.01b
W_{LDHA} after SPD	29.30 ± 0.30a	110.72 ± 1.42b	5.08 ± 0.16b	133.11 ± 1.51a	ND	52.80 ± 2.36a	331.00 ± 5.75a	NA

[a]Mean ± SD, $n = 3$. Values with different lower case letters in the same column for each SL are significantly different by Duncan's multiple-range test ($P < 0.05$). Average loss percentage of the tocopherol or tocotrienol is given in parentheses, $n = 3$. Values with different upper case letters in the same row are significantly different by Duncan's multiple-range test ($P < 0.05$). Abbreviations: T, tocopherol; T_3, tocotrienol; FFA, free fatty acid; SPD, short-path distillation; Sr, substrate; SDASO, NSL, LDHA, and DHASCO-FFAs: see Table 1 for explanation of abbreviations; W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively; ND, not detected; NA, not applicable.

RESULTS AND DISCUSSION

SLs were selected on the basis of enzymatic reaction types and lipase specificity. NSL was synthesized through enzymatic transesterification by enriching SDA soybean oil with about 60% palmitic acid esterified at the *sn*-2 position and about 8% of total SDA. NSL was also a starting substrate for LDHA, which further incorporated DHA by lipase-catalyzed acidolysis. These two SLs may be useful as human milk fat analogues with health benefits of omega-3 fatty acids. The FA profiles of substrates and resulting SLs after SPD are shown in Table 1. The results from the FA profile were used to estimate the average molecular weight of the substrates and products. More importantly, the fatty acid composition of the substrates determines the range of FA species that may be available to form tocopheryl and tocotrienyl fatty acid esters. The major FAs of SDASO were SDA (25.74 mol %), linoleic acid (24.22 mol %), palmitic acid (13.93 mol %), oleic acid (13.21 mol %), α-linolenic acid (11.01 mol %), γ-linolenic acid (7.22 mol %), and stearic acid (3.87 mol %). In the case of NSL, palmitic acid (67.49 mol %), linoleic acid (8.51 mol %), SDA (8.39 mol %), oleic acid (5.04 mol %), and stearic acid (2.54 mol %) were the dominant FAs, which were in agreement with our previous study.[16] The major FAs saponified from DHASCO were DHA (45.51 mol %), followed by oleic acid (23.07 mol %), myristic acid (10.94 mol %), and palmitic acid (7.45 mol %).

During lipase-catalyzed transesterification, FAs are cleaved off the glycerol backbone and not all of these FAs reattach to the TAG, resulting in FFAs. FFA contents of substrates and products are shown in Table 2. FFA percentage of NSL after reaction was higher than that of the original SDASO with a significant difference at $P < 0.05$. SPD removed most of the liberated FFAs and restored FFA content similar to fresh oil status (below 0.1%). For LDHA produced by acidolysis, a much higher FFA content (26.95%) was present in the product after reaction because DHASCO-FFAs were one of the starting substrates. Repetitive SPD was required to remove most of the FFA, but FFA percentage (0.35%) was not as low as substrate NSL ($P < 0.05$).

Tocopherol and tocotrienol concentrations of substrates, SLs before and after SPD, and distillates after SPD were measured by normal phase HPLC and are shown in Table 2. The elution order of tocopherols and tocotrienols was α-tocopherol, α-tocotrienol, β-tocopherol, γ-tocopherol, γ-tocotrienol, δ-tocopherol, and δ-tocotrienol. Due to the instability of these compounds, all of the test samples were protected from light and oxygen and analyzed on the day of preparation. According to the law of conservation of mass, loss percentages for different reactions (transesterification and acidolysis) as well as SPD were calculated, and average values were reported (Table 2). For both NSL and LDHA, more vitamin E isomers were lost after SPD than during enzymatic production. Specifically, there were 59 and 73% total losses of tocopherols and tocotrienols during purification by SPD compared to 3 and 21% losses during enzymatic production of NSL and LDHA, respectively. These lost tocopherols and tocotrienols after SPD were found to be present in the distillate at high concentration, which suggests that they should not be discarded as wastes. Instead, they should be recovered and added back to SLs to maintain or protect the oxidative stability of final products. As shown in Table 2, losses of α-, β-, γ-, and δ-tocopherols after SPD for LDHA were 61, 100, 86, and 88%, respectively, with 52, 51, 57, and 68% losses for NSL. A possible explanation for the higher

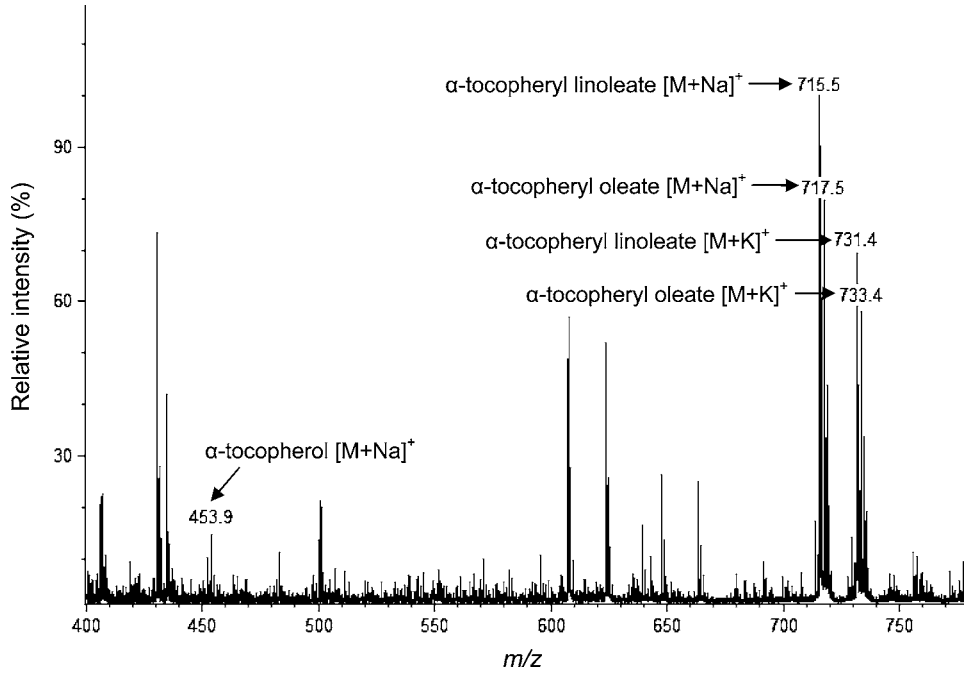

Figure 1. MALDI-TOF mass spectra of α-tocopherol, α-tocopheryl oleate, and α-tocopheryl linoleate from vitamin E linoleate mixture in positive ion mode.

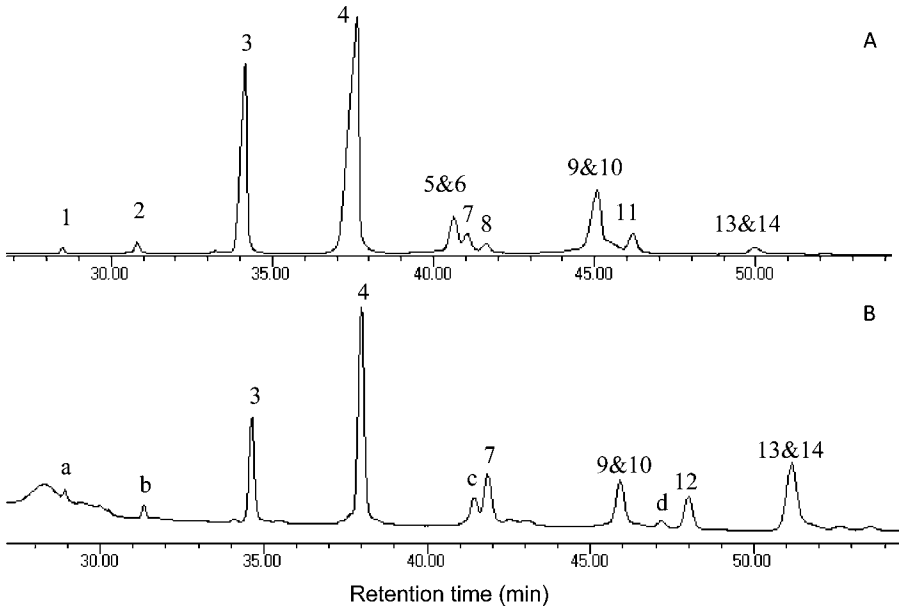

Figure 2. Total ion chromatograms of tocopheryl and tocotrienyl esters identified in the (A) W_{NSL} and (B) W_{LDHA} in the SIM mode. Peak numbers correspond to the esters listed in Table 3; a, b, c, and d are unknown compounds. (W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively.)

loss after SPD in LDHA than NSL may be due to repetitive passes to remove FFAs after acidolysis and therefore longer exposure to heat and light. Another observation was a higher loss of vitamin E isomers during acidolysis of LDHA (27, 20, 27, and 20% for α-, β-, γ-, and δ-tocopherols, respectively) compared to transesterification of NSL (5, 8, 1, and 7%). There are two possible explanations. First, longer reaction time under heat for LDHA (24 h) may contribute in part to the difference. Second, higher amounts of tocopheryl and tocotrienyl esters were estimated to have been formed by acidolysis than transesterification due to the availability of carboxylic acids. As can be seen in Table 2, for NSL and LDHA, there was a significant difference in loss percentage ($P < 0.05$) among tocopherol and tocotrienol species during either enzymatic reaction or purification. This may be possibly due to the preference of tocopherols or tocotrienols to form esters during transesterification and acidolysis. As mentioned above, these esters were removed into the distillate by SPD and contributed to the loss of tocopherols and tocotrienols. This may be partly attributed to relative sensitivity of these isomers in response to environmental stresses (e.g., oxygen, heat, light, and exposure time). For tocotrienols in LDHA, it was expected that

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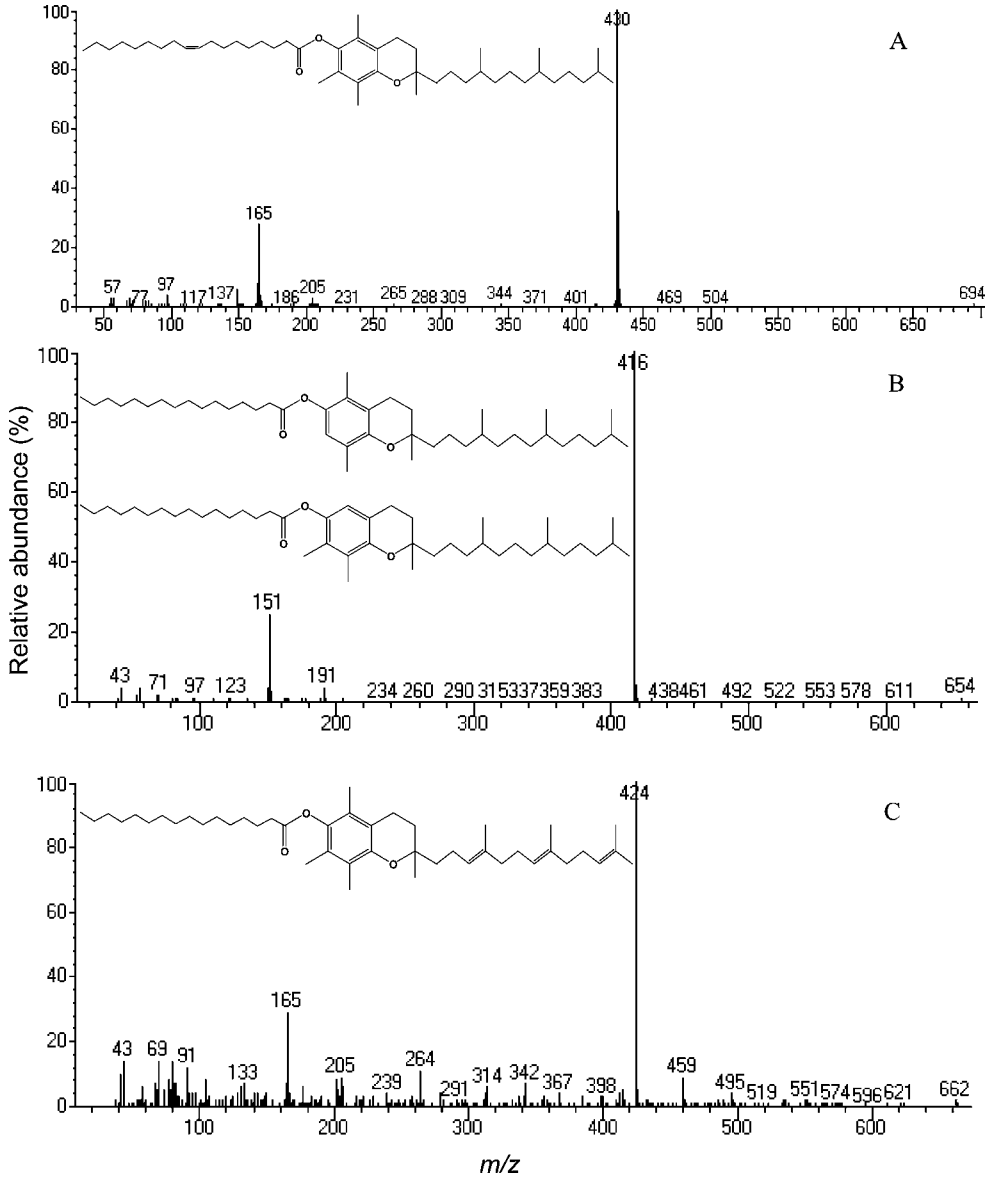

Figure 3. Representative structures and EI full scan mass spectra of tocopheryl and tocotrienyl esters: (A) α-tocopheryl oleate from vitamin E linoleate mixture; (B) β(or γ)-tocopheryl palmitate from W_{NSL}; (C) α-tocotrienyl palmitate from W_{LDHA}. (W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively.)

tocotrienols will degrade more than corresponding tocopherols during both acidolysis and SPD because of the presence of three unsaturated double bonds in the isoprenoid side chain, like γ-tocotrienol in this study. Surprisingly, α-tocotrienol had a much lower loss than α-tocopherol and other vitamin E isomers. The reason is unclear, and it is unlikely that the structural difference had any effect. Lee and Park[24] studied the stability of vitamin E isomers extracted from rice bran oil under various temperature and oxygen conditions. They found that α-tocotrienol degraded more rapidly than other isomers, whereas γ-tocopherol was the most stable isomer. Oxygen level also had significant impact on the stability of each isomer in their studies, where severe reductions in γ-tocopherol (by 20%) and γ-tocotrienol (29%) were observed under 2% oxygen conditions, whereas under 0% no degradation was observed even after exposure to 95 °C for 4 h.

Two mass spectrometric techniques, MALDI-TOF-MS and GC-MS, were employed to confirm the hypothesis that tocopheryl and tocotrienyl fatty acid esters were formed during both acidolysis and transesterification. A number of matrices were investigated for the MALDI analysis including 2,5-dihydroxybenzoic acid, α-cyano-4-hydroxycinnamic acid, and sinapinic acid. 2,5-Dihydroxybenzoic acid proved to work best in producing a mass profile of vitamin E linoleate mixture standard in a very clear and systematic manner (Figure 1). It can be seen that the spectra of α-tocopheryl oleate, α-tocopheryl linoleate, and α-tocopherol were dominated by sodiated molecules ($[M + Na]^+$) and showed no protonated molecules ($[M + H]^+$). α-Tocopheryl oleate and linoleate also yielded potassium adduct ions ($[M + K]^+$). After subtraction of the background and correction for isotope contributions, the data produced from distillates and residues of NSL and LDHA were identified on the basis of molecular masses, $[M + Na]^+$ and $[M + K]^+$, which were calculated according to the fatty acid and vitamin E isomer profiles of substrates. Unexpectedly, none of the ester ions were found in the spectra. This may be due to

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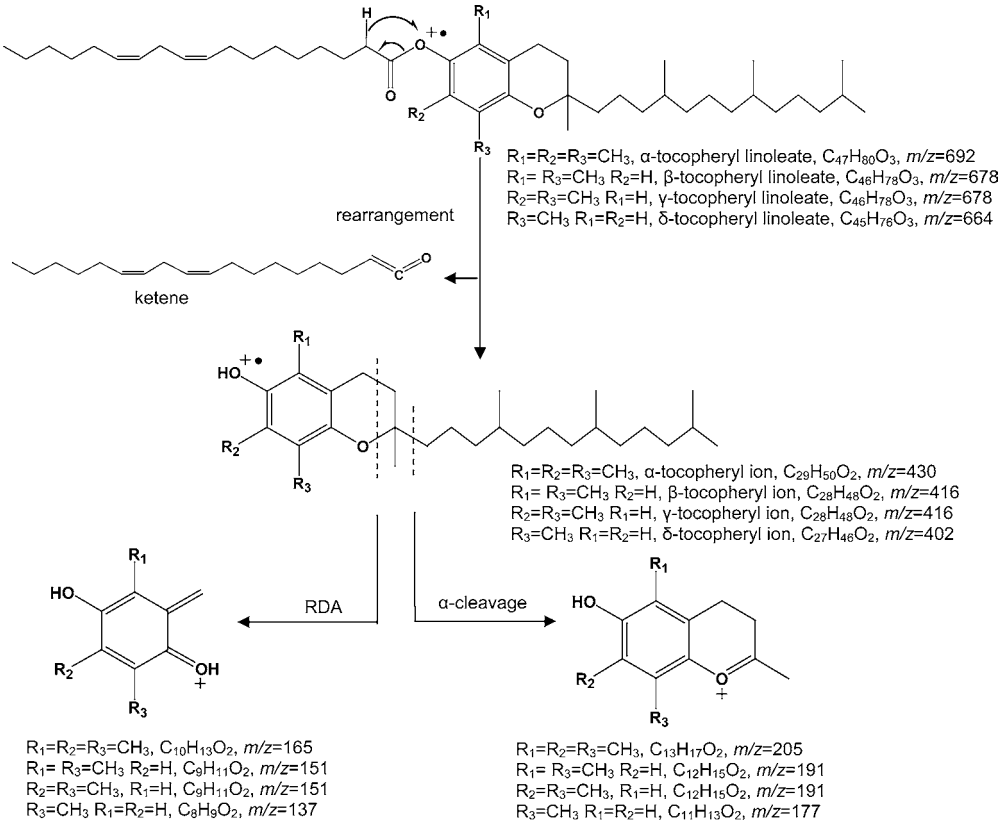

Figure 4. Mass spectrometric fragmentation mechanism of *α*-, *β*-, *γ*-, and *δ*-tocopheryl linoleate in the EI mode. (RDA, retro-Diels–Alder reaction.)

Table 3. Tocopheryl and Tocotrienyl Esters Identified by GC-MS in Distillates after Short-Path Distillation[a]

			GC-MS/EI[b]			GC-MS/PCI	W_{NSL}		W_{LDHA}	
no.[c]	tocopheryl/tocotrienyl ester	M_r	base peak	M^+	other ions	$[M + NH_4]^+$	EI	PCI	EI	PCI
1	*δ*-tocopheryl myristate	612	402 (100)	612 (2)	137 (18), 177 (9)	630	+	−	−	−
2	*β*(or *γ*)-tocopheryl myristate	626	416 (100)	626 (2)	151 (27), 191 (5)	644	+	−	−	−
3	*δ*-tocopheryl palmitate	640	402 (100)	640 (2)	137 (16), 177 (7)	658	+	+	+	+
4	*β*(or *γ*)-tocopheryl palmitate	654	416 (100)	654 (1.5)	151 (25), 191 (4)	672	+	+	+	+
5	*δ*-tocopheryl oleate	666	402 (100)	666 (1.5)	137 (17), 177 (8)	684	+	−	−	−
6	*δ*-tocopheryl linoleate	664	402 (100)	664 (1.5)	137 (19), 177 (8)	682	+	−	−	−
7	*α*-tocopheryl palmitate	668	430 (100)	668 (1.5)	165 (23), 205 (3)	686	+	−	+	+
8	*δ*-tocopheryl stearate	668	402 (100)	668 (1.2)	137 (17), 177 (6)	686	+	−	−	−
9	*β*(or *γ*)-tocopheryl oleate	680	416 (100)	680 (1.5)	151 (27), 191 (4)	698	+	+	+	−
10	*β*(or *γ*)-tocopheryl linoleate	678	416 (100)	678 (1.8)	151 (28), 191 (4)	696	+	+	+	−
11	*β*(or *γ*)-tocopheryl stearate	682	416 (100)	682 (1)	151 (26), 191 (3)	700	+	−	−	−
12	*α*-tocotrienyl palmitate	662	424 (100)	662 (3)	165 (28), 205 (12)	680	−	−	+	−
13	*α*-tocopheryl oleate	694	402 (100)	694 (1)	165 (27), 205 (4)	712	+	−	+	+
14	*α*-tocopheryl linoleate	692	402 (100)	692 (1)	165 (28), 205 (4)	710	+	−	+	+

[a]Abbreviations: M_r, molecular weight; W_{NSL} and W_{LDHA}, see Table 2 for explanation of abbreviations; M^+ and $[M + NH_4]^+$, molecular ion formed by EI and PCI, respectively; +, detected; −, not detected. [b]Characteristic ions formed by EI and their relative abundance (%) (in parentheses). [c]Number corresponds to the numbers in Figure 2.

a strong "matrix effect" from abundant endogenous compounds, such as TAGs, diacylglycerols, monoacylglycerols, and FFAs, which interfere with tocopheryl and tocotrienyl fatty acid esters present at much lower levels.

However, this challenge was overcome by selection of GC-MS in synchronous scan/SIM mode to achieve column baseline separation and mass spectrometric separation as well. Synchronous scan/SIM is an acquisition technique by rapid alternation between a full scan and SIM modes, which enables simultaneous collection of common full scan data (qualitative) and SIM data (quantitative) in a single run without sacrificing performance.[25] The SIM mode offers significant improvement in sensitivity over full scan mode; thus, it can be utilized to track signals of trace level compounds by eliminating the matrix noise. Because the data were acquired simultaneously, the uncertainty in compound identification was minimized and sample analysis time was reduced. Due to high molecular weight and boiling points of tocopheryl and tocotrienyl esters, high column temperature was used to separate these compounds. Total ion chromatograms of identified tocopheryl

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and tocotrienyl esters in the distillates of NSL and LDHA in the SIM mode and some typical full scan mass spectra are presented in Figures 2 and 3, respectively. The peak order of esters was δ-, β(or γ)-, and α-moiety, separately. Tocopheryl/tocotrienyl fatty acid esters have their own unique EI fragmentation patterns. Taking tocopheryl linoleate as an example (Figure 4), after ionization, the molecular ion of tocopheryl linoleate undergoes a rearrangement involving hydrogen transfer from α-carbon to ester oxygen to remove the linoleic acid moiety. The resulting fragments include a neutral ketene molecule and a radical cation of the phenyl alcohol at m/z 430, 416, 416, and 402, respectively (α-, β-, γ-, and δ-tocopheryl linoleate, respectively). The tocopherol ion (base peak) is further fragmented by retro-Diels–Alder reaction with hydrogen transfer into an intense ion at m/z 165, 151, 151, and 137, respectively, and also by α-cleavage into a weaker ion at m/z 205, 191, 191, and 177, respectively. The β- and γ-isomers may not be discriminated because they have the same number of methyl groups at different positions in the chroman ring (Figure 4). The fragmentation mechanism was supported by direct analysis of vitamin E linoleate mixture standard (Figure 3) and previous studies.[26,27] Similarly, tocotrienyl esters undergo the same fragmentation routes as tocopheryl esters, creating different tocotrienyl ions (base peak) but the same secondary fragmented ions (Figure 3). As a result, these characteristic fragment ions were chosen in synchronous scan/SIM mode to track and distinguish different types (α, β, γ, or δ) of tocopheryl/tocotrienyl moieties. Finally, α-, β-, γ-, and δ-tocopheryl/tocotrienyl fatty acid esters, although very low in the samples, could be identified on the basis of additional information from observed molecular ions and theoretical molecular mass calculation. Meanwhile, none of these esters were found in the residues of either NSL or LDHA, which indicates that all of the tocopheryl and tocotrienyl esters formed during acidolysis and transesterification were fractionated into distillates after SPD.

Furthermore, under the same analytical conditions, GC-MS/PCI was employed to verify the identification of the tocopheryl and tocotrienyl esters in the distillates, the molecular ions of which were produced at extremely weak abundance in EI mode (Figure 3). PCI is a soft ionization technique, and pseudomolecular ion, $[M + NH_4]^+$, is yielded at relatively high intensity when using ammonia as the reagent gas. Tocopheryl and tocotrienyl fatty acid esters identified by GC-MS in EI and PCI modes are summarized in Table 3. For NSL by transesterification, δ- and β(or γ)-tocopheryl palmitate, β(or γ)-tocopheryl oleate, and β(or γ)-tocopheryl linoleate were confirmed in both EI and PCI modes, whereas δ- and β(or γ)-tocopheryl myristate, δ- and α-tocopheryl oleate, δ- and α-tocopheryl linoleate, α-tocopheryl palmitate, and δ- and β(or γ)-tocopheryl stearate were confirmed only in EI mode. In the case of LDHA by acidolysis, δ- and β(or γ)-tocopheryl palmitate, α-tocopheryl palmitate, α-tocopheryl oleate, and α-tocopheryl linoleate were verified in PCI and EI modes, whereas β(or γ)-tocopheryl oleate, β(or γ)-tocopheryl linoleate, and α-tocotrienyl palmitate were verified only in EI mode. The dominant esters formed were tocopheryl palmitate, tocopheryl oleate, and tocopheryl linoleate homologues, which were found in both NSL and LDHA. However, tocopheryl myristate and stearate and δ-tocopheryl linoleate and oleate were identified only in NSL even though a higher content of corresponding fatty acids was present in the reaction medium of LDHA (Table 1), and α-tocotrienyl palmitate was solely formed in LDHA. It is likely that vitamin E isomers in the reaction mixture, not FFA, primarily play a role in the formation of tocopheryl and tocotrienyl fatty acid esters. It is also important to note that none of the tocopheryl/tocotrienyl linolenate, stearidonate, and docosahexaenoate were identified in either NSL catalyzed by nonspecific lipase or LDHA catalyzed by *sn*-1,3 specific lipase. This may be due to the preference of lipases, Lipozyme TL IM and Novozym 435, for a specific fatty acid or fatty acids with a certain chain length range and unsaturation.[28] Peng et al.[29] reported similarly that Lipozyme TL IM showed a slight discrimination over very long chain polyunsaturated fatty acids, eicosapentaenoic acid and DHA, compared to conjugated linoleic acid and caprylic acid.

The assumption that tocopheryl and tocotrienyl fatty acid esters are formed during transesterification in addition to acidolysis was successfully confirmed. All of the esters were fractionated into distillates after SPD. This is the first report that determined these trace-level esters in distillates by GC-MS in EI and PCI modes on the basis of synchronous scan/SIM acquisition technique. Furthermore, >50% of vitamin E isomers were lost into distillates during SPD, which contributed mostly to the rapid oxidative deterioration of SLs in the past studies and to a lesser extent from the formation of tocopheryl and tocotrienyl esters. This implies that these lost tocopherols and tocotrienols in the distillates, previously discarded as wastes, may be recovered and even added back to the final oil products (SLs) to improve their oxidative stability.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

SL(s), structured lipid(s); triacylglycerols, TAGs; FA(s), fatty acid(s); FFA(s), free fatty acid(s); SPD, short-path distillation; SDASO, stearidonic acid soybean oil; DHASCO, docosahexaenoic acid single-cell oil; DHA, docosahexaenoic acid; NSL, structured lipid of SDASO and tripalmitin catalyzed by Novozym 435; LDHA, structured lipid of DHA and NSL catalyzed by Lipozyme TL IM; DHASCO-FFAs, free fatty acids hydrolyzed from docosahexaenoic acid single-cell oil; W_{NSL} and W_{LDHA}, distillate by short-path distillation from NSL and LDHA, respectively

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